Biochemical and molecular approach to cephalopod phylogeny

Renata Boucher-Rodoni and Laure Bonnaud

Biologie des Invertébrés marins et Malacologie, URA 699 and GDR 1005 CNRS, Muséum national d'Histoire naturelle, 55 rue Buffon, 75005 Paris, France

Abstract: Cephalopod taxonomy is still uncertain, and little is known of the phylogeny of Recent taxa. Biochemical and molecular characters are complementary to morphology, and allow an additional insight into the phylogenetic relationships among cephalopods. Eye lens protein electrophoresis and immunological approaches yield data in agreement with traditional taxonomic grouping, but are less suitable for establishing phylogenetic relationships. Molecular tools, *e. g.* the 3' end of the 16S rDNA gene, have failed to resolve the phylogeny at the suprafamilial level, but seem appropriate at lower levels. DNA sequence comparisons (% substitution) show that a direct relationship between taxonomic rank and nucleotide divergence cannot be established, as the nucleotide divergence level differs from one taxa to the other.

Electrophoretic and immunological analyses of eye lens proteins as well as molecular results suggest that sepiolids should be separated from Sepioidea.

Phylogenetic analyses allow hypotheses of biological evolution based on various criteria to be tested. Morphological classification of taxa has, historically, been the first approach considered, and the analyses of anatomical structures are still of prime importance in the construction of hypotheses on the evolutionary history of organisms. But more recently, the development of biochemical and molecular techniques, that allow an insight into genetic structure, have opened new perspectives in taxonomy and phylogeny. Paleontology provides, however, the only direct means of calibrating evolutionary events. Both paleontology and embryology allow recognition of homologous characters derived from a common ancestor, the ancestors being deduced from the characters of terminal groups.

As far as cephalopods are concerned, the taxonomic status of many groups is still uncertain, and little is known of the phylogeny of Recent taxa. The main reason for this is that although the fossil record contains a wealth of ectocochleate forms (all extinct except *Nautilus*), this is not the case for Recent, mainly soft-bodied animals with a reduced or absent internal shell. So far only a few authors have considered the phylogenetic systematics of the group Cephalopoda. Fioroni (1981) was one of the first to use the Hennig (1966) approach to systematics. Berthold and Engeser (1987) established adelphotaxonomic relationships by the identification of synapomorphies in fossil and Recent taxa and proposed a phylogenetic classification of 35 subordinate taxa. The cladograms presented in these papers are not totally in agreement, drawing attention to the necessity of using additional criteria, such as biochemical and molecular, for taxonomic and phylogenetic reconstruction of cephalopod systematics.

All approaches, morphological, biochemical, and molecular, are complementary but each has advantages and disadvantages. Molecular analyses provide new tools to test the hypotheses based on morphology, and help to reformulate them in some cases. Morphological characters are more accessible, easier and less costly to analyze, allow comparison of extant and fossil forms, but can be more subjective. Biochemical and molecular characters are more objective, are potentially very abundant, but are not always easy to analyze. For instance, the fact that the nucleotides at each position can exist in only four states could be an important source of homoplasy. It is also well known that different portions of the same gene have not all the same probability of variation, and the rate of evolution can be different for the same gene in different taxa.

This paper is aimed to compare the results obtained using biochemical (electrophoretic and immunological) and molecular techniques for phylogenetic reconstruction of cephalopods using a number of species, comprising octopods and decapods.

MATERIALS AND METHODS

Fresh cephalopod tissue samples were obtained from various sources (Table 1). They were either stored at -20°C prior to electrophoretic and immunological analyses, or alcohol preserved for nucleotide sequencing.

Table 1. List of cephalopod species analyzed by eye lens protein electrophoresis and mtDNA sequencing. Their geographical origin is indicated as well as the source of the data: 1, Bonnaud *et al,* 1994; 2, Tranvouez and Boucher-Rodoni, 1990; X, present paper; (-), no data.

Origin	Species	Eye lens	mtDNA
East Atlantic (Biscay)	*Sepia officinalis* Linné, 1758	X	1
Mediterranean (Banyuls)	*S. orbignyana* Férussac, 1826	2	1
SW Pacific (New Caledonia)	*S. latimanus* Quoy and Gaimard, 1832	X	1
English Channel (Roscoff)	*Loligo vulgaris* Lamarck, 1798	2	1
SW Pacific (New Caledonia)	*Sepioteuthis lessoniana* Lesson, 1830	X	1
English Channel (Roscoff)	*Sepiola atlantica* Orbigny, 1840	X	1
Mediterranean (Banyuls)	*Rossia macrosoma* (Delle Chiaje, 1829)	X	1
Mediterranean (Banyuls)	*Todaropsis* sp.	X	(–)
Pacific Ocean (Hawaii)	*Todarodes* sp.	(–)	1
Mediterranean (Banyuls)	*Octopus vulgaris* Cuvier, 1797	2	(–)
Mediterranean (Banyuls)	*Eledone cirrhosa* (Lamarck, 1798)	2	1
SW Pacific (New Caledonia)	*O. cyanea* Gray, 1849	X	1
SW Pacific (New Caledonia)	*O.* sp.	X	(–)
East Atlantic (Mauritania)	*Graneledone verrucosa* (Verrill, 1881)	X	(–)
East Atlantic (Mauritania)	*Opistoteuthis agassizii* (Verrill, 1883)	X	(–)

For biochemical approaches (electrophoresis and immunology), the protein chosen should be stable enough that individual physiological changes do not influence the observed differences between species, but it should be variable enough to reflect taxonomic and phylogenetic differences. Eye lens proteins and hemocyanin were tested here.

Eye lens proteins were extracted according to the protocols described in Tranvouez and Boucher-Rodoni (1990), and analyzed on precast polyacrylamide gels (ExcelGel SDS Gradient 8-18%, Pharmacia). A band presence/absence matrix was computed and processed by the NTSYS-pc program (Rohlf, 1990), using Sahn clustering (Sneath and Sokal, 1973) with UPGMA and Neighbor-Joining (NJ) methods (Saitou and Nei, 1987), to produce phenograms. PAUP 3.1 (Swofford, 1985) was used to estimate phylogenetic trees.

Protein antigenic properties are supposed to allow the estimation of immunological distance between taxa (Tsusumi *et al.*, 1989). The ELISA immunological technique, an enzyme-linked immunosorbent assay, was adapted to cephalopod eye lens protein (Boucher-Rodoni *et al.*, 1995). Four eye lens antisera were available (*Sepia officinalis, S. orbignyana, Loligo vulgaris, Octopus vulgaris*). A homologous standard inhibition curve was determined with 2500-fold diluted serum, and the affinity of heterologous samples was then tested.

The antigenic properties of hemocyanin, the large respiratory protein found in the blood of all cephalopods, was also used here to estimate taxonomic relationships. The immunological distance of various cephalopod species was estimated by heterologous reaction against commercial keyhole limpet hemocyanin (KLH) and compared to preliminary results obtained with homologous antiserum.

For molecular analyses, attention was first focused on mitochondrial DNA (mtDNA) because of its diversity, and because data on various groups, including non-molluscan invertebrates, are well known. mtDNA is a small circular DNA molecule present in many copies in the mitochondria, it is maternally inherited, and there is no recombination. The protein genes and the r-RNA genes have both a mosaic structure of conserved and variable regions, which should allow phylogenetic relationships at various hierarchical levels to be analyzed. Nucleotide sequence data from the 3' end of the 16S rDNA gene have already been used to analyze phylogenetic relationships among decapod cephalopods (Bonnaud *et al.*, 1994). To determine whether the different taxonomic hierarchical levels can be related to a given nucleotide percentage of divergence, some sequences from two populations of the same species, from different species of the same genus, and from different genera, were compared and analyzed in terms of molecular divergence (% substitution).

RESULTS

The results of eye lens protein electrophoresis of 14 cephalopod species analyzed by UPGMA and Neighbor-Joining methods show that with both methods the taxonomic grouping is respected (Fig. 1). Octopods are always grouped together, but are not distinctly separated from decapods. Incirrate octopods are a sister group of cirrate octopods, but at lower taxonomic levels the genus *Octopus* is not homogeneous. As far as decapods are concerned, the relationships among myopsids, oegopsids, and sepioids are not clearly defined by either approach. The sepiolids occupy a particular position in the NJ analysis, branching as a sister group of all other coleoids. The strict consensus tree derived from the phylogenetic analysis (PAUP 3.1) is less

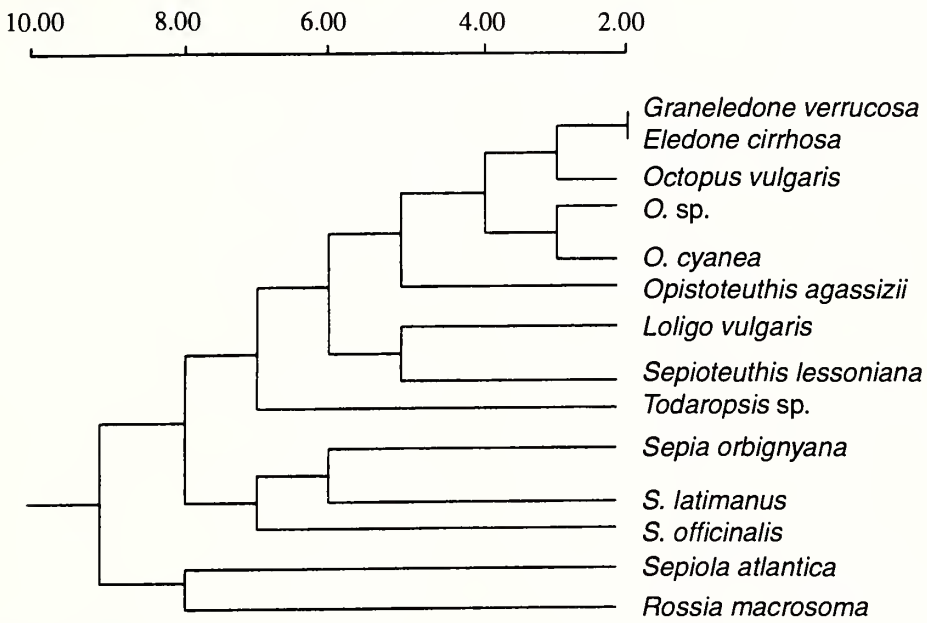

Fig. 1. Phylogenetic tree obtained by the Neighbor-Joining method on SDS-Page electrophoretic analysis of eye lens proteins.

resolved for octopods (Fig. 2).

When using protein eye lens immunological properties to estimate immunological distances for phylogenetic analyses, one of the main problems encountered was that in some cases the distances were not symmetrical. Table 2 shows that when comparing the heterologous reaction between two species using each species extract in turn as antiserum and antigen, the immunological distance is not necessarily the same.

The hemocyanin is currently used as an immunogenic agent. In cephalopods it is composed of seven functional units in octopods and *Nautilus*, and eight functional units in decapods (Van Holde *et al.*, 1992). Fig. 3 shows the immunoreactivity of the hemocyanin of various cephalopod species against KLH antiserum, estimated by the ELISA technique. *Nautilus* reactivity was the closest to KLH homologous reaction, most of the other species being grouped together at a rather distant position, except *Sepia* which displayed a reactivity stronger than all other coleoids. Preliminary assays with homologous hemocyanin antiserum indicate that the distances are readable at high hierarchical levels (*i. e.* distant taxa), but do not discriminate species. The distance between *Sepia* and *Nautilus* was the smallest, but the difference was not as important as with KLH. And again, as with eye lens proteins, the results were not symmetrical.

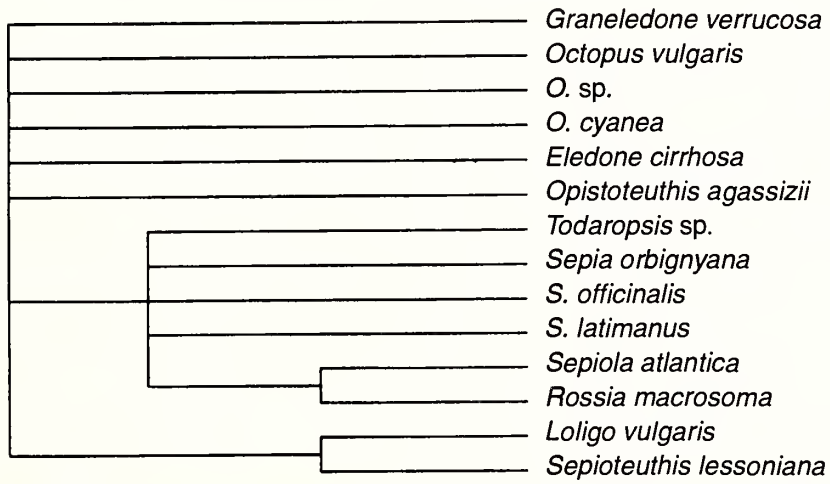

Fig. 2. Strict consensus tree (unrooted) of 100 trees obtained by heuristic search (PAUP 3.1; MULPARS option).

Table 2. Results of immunological heterologous reaction (ELISA technique) between species pairs using each species extract in turn as antiserum and antigen. Values represent immunological distances estimated by optical density differences.

ANTISERUM		ANTIGEN
Sepia officinalis	4 / 4	*S. orbignyana*
S. officinalis	10 / 15	*Loligo vulgaris*
L. vulgaris	17 / 13	*S. orbignyana*
Octopus vulgaris	16 / 25	*S. officinalis*
O. vulgaris	15 / 17	*S. orbignyana*
O. vulgaris	24 / 25	*L. vulgaris*

To analyze the relationships of cephalopods at a perispecific level (population, species), many authors have studied enzymatic polymorphism which remains a valuable tool for genetic population analyses (Levy *et al.*, 1988; Carvalho *et al.*, 1992; Brierley *et al.*, 1993, 1995).

If mtDNA is considered, preliminary results on six different populations of *Sepia officinalis* indicate that neither 16S, nor cytochrome oxidase CoII or CoIII, displays adequate variability for taxonomic purposes, whereas enzymatic polymorphism does (Bonnaud, unpub. data). Accordingly, when comparing the 3' end of 16S rDNA gene sequences, in terms of % substitution, the difference between two distant populations of *S. officinalis* (Mediterranean and English Channel) is not significant (Table 3). Therefore, this gene portion is not appropriate to provide evidence of differences among *S. officinalis* populations.

At higher taxonomic levels, the status of many cephalopod groups is still uncertain and needs to be reconsidered with the help of phylogenetic reconstruction. Phylogenetic hypotheses to estimate divergence time are usually given through paleontology (Doyle *et al.*, 1994). True cuttlebones are only known from the early Tertiary; Teuthoidea and Sepioidea are thus supposed to have diverged in the Cenozoic.

The use of mtDNA 16S for phylogenetic reconstruction was shown to be appropriate to test Tertiary divergences in insects and vertebrates (Simon *et al.*, 1990; Hillis and Dixon, 1991), but such is not the case for cephalopods (Bonnaud *et al.*, 1994). The phylogeny of cephalopods is unresolved at the suprafamilial level because of excessive nucleotide divergence (saturation), perhaps due to earlier emergence than Cenozoic, or to unequal evolutionary rates among taxa.

To compare results derived from protein elec-

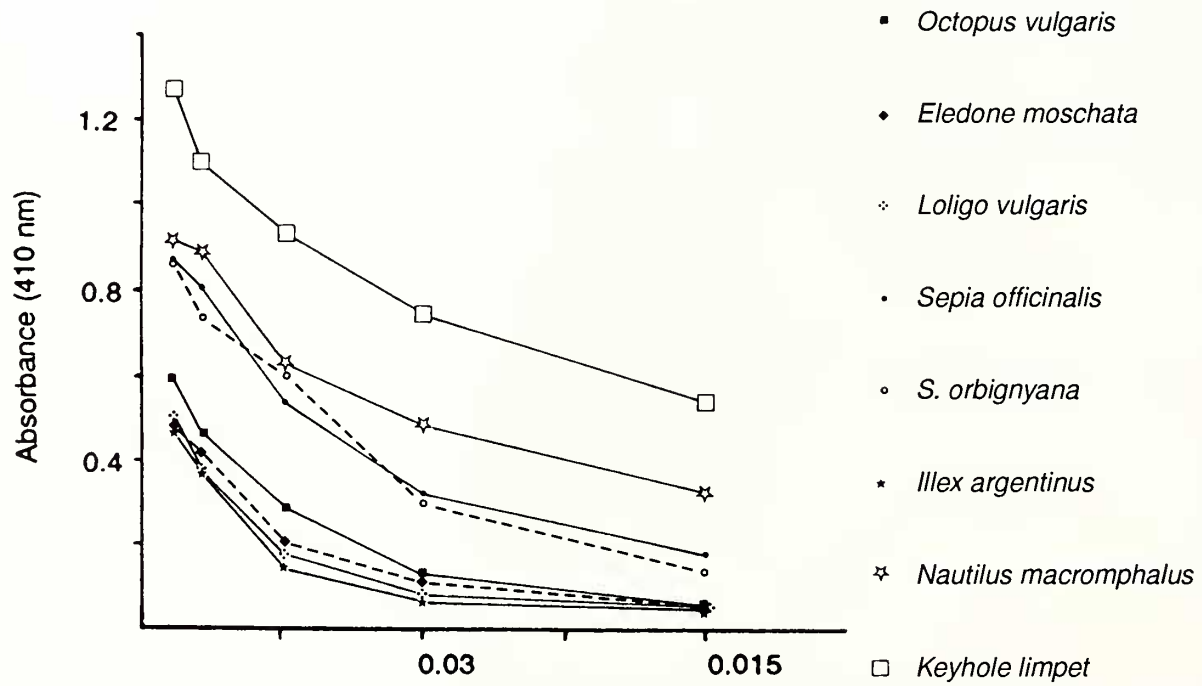

Fig. 3. Protein (µg/ml) Immunoreactivity of hemocyanin of various cephalopod species against keyhole limpet hemocyanin antiserum, tested by the ELISA technique.

Table 3. Nucleotide divergence of 3' end of 16S rDNA gene sequences at various taxonomic levels: (a) populations, (b) intrageneric, (c) intergeneric.

	Species	Divergence (% substitution + gaps)
(a)	*Sepia officinalis* (Roscoff) - *S. officinalis* (Banyuls)	*ca.* 1
(b)	*S. officinalis* - *S. orbignyana*	13.8
	S. orbignyana - *S. elegans*	8.1
	S. elliptica - *S. pharaonis*	10.3
	S. pharaonis - *S. smithi*	8.8
	Loligo forbesi - *L. vulgaris*	9.4
	Nautilus macromphalus - *N. pompilius*	5.5
(c)	*Sepietta* sp. - *Sepiola atlantica*	5.3
	Sepietta sp. - *Rossia macrosoma*	13.0
	Sepiola atlantica - *Rossia macrosoma*	12 1
	Sepietta sp. - *Sepia officinalis*	17.9
	Sepietta sp. - Loligo vulgaris	18.4*

*Overestimated value, because of an insertion of *ca.* 20 bases in *Loligo*.

trophoresis and sequence data from the 3' end of 16S rDNA, the sequences corresponding to some of the taxa appearing in Fig. 1 were aligned and analyzed by Neighbor-Joining and PAUP methods. Both approaches show that nucleotide analysis clearly separates octopods and decapods, but again the monophyly of the order Sepioidea, including sepiids and sepiolids is not supported, the sepiolids being excluded from the order (Fig. 4). In terms of % substitution, sequence comparison shows that sepiolids are as distant from *Sepia* (17.9%) as from *Loligo* (18.4%) (Table 3). Intrageneric divergence ranges from 8 to 14% for sepiids (the highest value concerning *S. officinalis* and *S. orbignyana*), but a direct relationship between taxonomic rank and nucleotide divergence cannot be established, as the nucleotide divergence level in other taxa could be in another range. Between sepiolid genera, for instance (Table 3), the intergeneric divergence is 5.3% between *Sepiola* and *Sepietta*, two morphologically closely related species, but can be as high as 13.0% between *Sepietta* and *Rossia*. One surprising result concerns the sequence comparison of two morphologically distinct species of *Nautilus* which display a low nucleotide divergence value (5.5%).

DISCUSSION AND CONCLUSION

The reliability of eye lens proteins as a taxonomic tool was shown by some authors (Smith, 1969; Swanborn, 1971; Brahma and Lancieri, 1979; Tranvouez and Boucher-Rodoni, 1990). It is confirmed here that eye lens protein electrophoresis analysis serves to group closely related taxa together, but its use for inferring phylogenetic relationships leads to more questionable results. The use of immunological properties of eye lens proteins and of hemocyanin to analyze taxonomic relationships is rather disappointing, mainly because the distances measured by the ELISA technique between taxa are not symetrical, *i. e.* the distance between *Sepia* and *Nautilus* is not the same as the distance between *Nautilus* and *Sepia*. The immunological distance indicates the degree of similarity, but it is not a character that is easy to precisely quantify for phenetic or phylogenetic analyses.

The development of molecular biology has raised great hope and excitement about phylogenetic reconstructions. We now have direct access to the genetic material. However the enormity of the available genetic information is itself a problem: where is the most appropriate place to investigate to answer our questions? Rates of evolution differ from one gene to the other, and the main problem is to find a genetic marker appropriate for the hierarchical level we are interested in. If a gene has evolved too rapidly, it will be saturated with substitutions and provide a non-significant result. If it is too stable, the variability will not be informative enough. Our knowledge of genetic structures comes primarily from results obtained with vertebrates or *Drosophila*, but their levels of nucleotide divergence are not necessarily adequate for analysis of other phylogenetic relationships. This cannot be known *a priori*, and a series of prerequisites are necessary before starting a molecular analysis. (1) Choice of the gene: its variability must be adequate to the hierarchical level being considered. (2) Choice of the species (number and "quality"): this is important because some cephalopod genera include over 100 species, whereas others are monotypic. (3) Length of the sequence: apparently, bootstrap values increase with length of sequence, but the reliability depends rather on the number of taxa (Lecointre *et al.*, 1993). So, as with morphology, we will have to increase, as much as possible, the number of species for each taxon. This is obviously impossible when some genera or even families are monospecific. (4) Choice of an outgroup: it should comprise more than one species of a monophyletic group, close enough to the investigated taxon to preclude saturation, but sufficiently different to prevent inclusion in the ingroup.

As far as cephalopods are concerned, nucleotide sequence analysis provides a more reliable picture than electrophoresis and immunology which are interesting taxonomic tools, but are not satisfactory for phylogenetic analyses. However, a direct relationship between taxonomic rank and % nucleotide divergence cannot be established, as the nucleotide divergence level is different in different taxa.

The congruence between morphological and molecular analyses, two independent sets of data, is very important in construction of evolutionary patterns. Morphological-molecular comparisons however are still rare, and

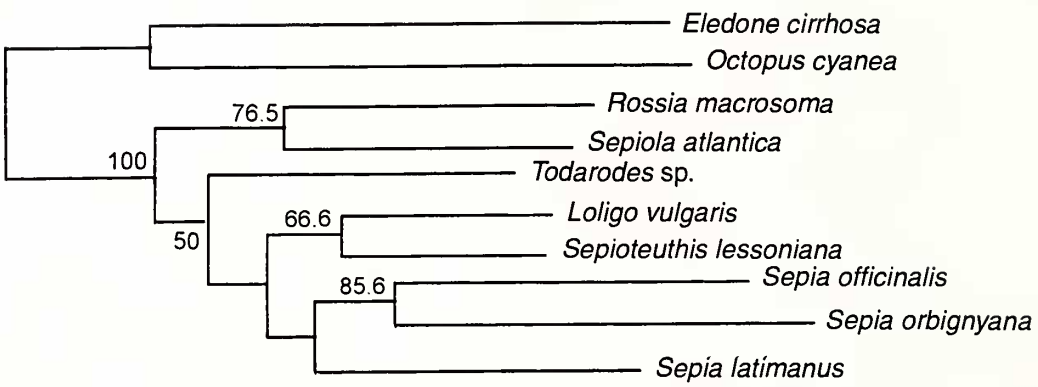

Fig. 4. Phylogeny inferred by Neighbor-Joining distance analysis from mtDNA 16S sequences (Bonnaud *et al*, 1994). Bootstrap values ≤ 50 are indicated.

not often congruent. In cephalopods, one solid and congruent result concerns sepiolids. Their taxonomic and phylogenetic position has been a matter of much discussion, being classified either as a family of the Sepioidea (Naef, 1912; Voss, 1977; Mangold and Portman, 1989) or as a family of the Myopsida together with the Sepiidae (Berthold and Engeser, 1987). Fioroni (1981, based on embryology) and Clarke (1988, based on morphology) proposed to raise the sepiolids to ordinal rank. The present electrophoretic, immunological, and molecular results confirm that sepiolids can be separated from the Sepioidea.

The analysis of phylogenetic relationships among coleoids must take into account a number of difficulties, whatever the criteria used, morphological, biochemical, or molecular: (1) poor fossil remains of recent taxa; (2) no outgroup: we choose octopods as an outgroup for decapods, and *vice versa* because, even if the distance is very important, we have no real alternative; (3) many monospecific genera; (4) traditional classification not well stabilized; (5) little information on rate and modalities of molecular evolution: the evolutionary rate of the different genes is inferred mainly from vertebrate results - vertebrates represent only one episode of the saga of life and it is not always possible to transpose to invertebrates genetic postulates based on vertebrate results.

To better understand higher hierarchical levels of phylogeny, it appears necessary in many cases to consider more than one gene, preferably by associating mitochondrial and nuclear information.

ACKNOWLEDGMENTS

The authors wish to thank all the colleagues who helped in gathering specimens from different parts of the world, and particularly Drs. C. C. Lu, R. E.Young, and S. v. Boletzky. The technical support of Mrs. M. Martin was greatly appreciated.

LITERATURE CITED

Berthold, T. and T. Engeser. 1987. Phylogenetic analysis and systematization of the Cephalopoda (Mollusca). *Verhandlungen des naturwissenschaftlichen Vereins Hamburg* (NF) 29:187-220.

Bonnaud, L., R. Boucher-Rodoni, and M. Monnerot. 1994. Phylogeny of decapod cephalopods based on partial 16S rDNA nucleotide sequences. *Comptes Rendus de l'Académie des Sciences, Paris, Sciences de la Vie* 317:581-588.

Boucher-Rodoni, R., M. Martin, and R. Peduzzi. 1995. Preliminary results of a comparative study on antigenicity of cephalopod eye-lens proteins: the sepiolids. *Bulletin de l'Institut Océanographique, Monaco* 16:93-98.

Brahma, S. K. and M. Lancieri. 1979. Isofocusing and immunological investigations on cephalopods lens proteins. *Experimental Eye Research* 29:671-678.

Brierley, A. S., P. G. Rodhouse, J. P. Thorpe, and M. R. Clarke. 1993. Genetic evidence of population heterogeneity and cryptic speciation in the ommastrephid squid *Martialia hyadesi* from the Patagonian shelf and Antarctic Polar frontal zone. *Marine Biology* 116:593-602.

Brierley, A. S., J. P. Thorpe, G. J. Pierce, M. R. Clarke, and P. R. Boyle. 1995. Genetic variation in the neritic squid *Loligo forbesi* (Myopsida: Loliginidae) in the northeast Atlantic Ocean. *Marine Biology* 122:79-86.

Carvalho, G. R., A. Thompson, and A. L. Stoner. 1992. Genetic diversity and population differentiation of the shortfin *Illex argentinus*. *Journal of Experimental Marine Biology and Ecology* 158:105-121.

Clarke, M. R. 1988. Evolution of buoyancy and locomotion in recent cephalopods. *In: The Mollusca. Vol. 12, Paleontology and Neontology of Cephalopods,* M. R. Clarke and E. R. Trueman, eds. pp. 203-213. Academic Press, New York.

Doyle, P., D. T. Donovan, and M. Nixon. 1994. Phylogeny and systematics of the Coleoidea. *University of Kansas Paleontological Contributions* 5:1-15.

Fioroni, P. 1981. Die Sonderstellung der Sepioliden, ein Vergleich der Ordnungen der rezenten Cephalopoden. *Zoologische Jahrbücher, Systematik* 108:178-228.

Hennig, W. 1966. *Phylogenetic Systematics.* University of Illinois Press, Urbana. 263 pp.

Hillis, D. M. and M. T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology*

66(4):411-453.

Lecointre, G., H. Philippe, and H. Le Guyader. 1993. Species sampling has a major impact on phylogenetic inference. *Molecular Phylogenetics and Evolution* 2(3):205-224.

Levy, J. A., M. Haimovici, and M. Conceicao. 1988. Genetic evidence for two species of the genus *Eledone* (Cephalopoda: Octopodidae) in south Brazil. *Comparative Biochemistry and Physiology* 90B(2):275-277.

Mangold, K. and A. Portman. 1989. Systématique. *In: Traité de Zoologie.* P. P. Grassé, ed. pp. 643-713. Masson et cie, Editeurs, Paris.

Naef, A. 1912. Teuthologische Notizen. 2. Die Gattungen der Sepioliden. *Zoologischer Anzeiger* 39: 244-248.

Rohlf, F. J. 1990. *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System.* Exeter Software, New York.

Saitou, N. and M. Nei. 1987. The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.

Simon, C., S. Pääbo, T. D. Kocher, and A. C. Wilson. 1990. Evolution of mitochondrial ribosomal RNA in insects as shown by the polymerase chain reaction. *In: Molecular Evolution,* M. Clegg and S. O'Brien, eds. UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 122. pp. 235-244. Wiley-Liss, New York.

Smith, A. C. 1969. An electrophoretic study of proteins extracted in distilled water and in saline solution, from the eye lens nucleus of the squid, *Nototodarus hawaiiensis* (Berry). *Comparative Biochemistry and Physiology* 30: 551-559.

Sneath, P. H. A. and R. R. Sokal. 1973. *Numerical Taxonomy.* Freeman, San Francisco. 573 pp.

Swanborn, P. L. 1971. Cephalopod lens proteins. *Experimental Eye Research* 11:111-115.

Swofford, D. L. 1985. *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.* Illinois Natural History Survey, Champaign, Illinois.

Tranvouez, Y. and R. Boucher-Rodoni. 1990. Eye lens SDS-Page electrophoresis and the systematics of cephalopods. *Cahiers de Biologie Marine* 31:281-289.

Tsusumi, H., S. Nakamura, K. Sato, K. Tamaki, and Y. Katsumata. 1989. Comparative studies on an antigenicity of plasma proteins from humans and apes by ELISA: a close relationship of chimpanzee and human. *Comparative Biochemistry and Physiology* 94B:647-649.

Van Holde, K. E., K. I. Miller, and W. H. Lang. 1992. Molluscan hemocyanins: structure and function. *In: Advances in Comparative and Environmental Physiology,* Vol. 13. pp. 257-300. Springer Verlag, Berlin and Heidelberg.

Voss, G. L. 1977. Present status and new trends in cephalopod systematics. *In: The Biology of Cephalopods,* M. Nixon and J. B. Messenger, eds. pp. 49-60. Symposium of the Zoological Society of London, 38.

Date of manuscript acceptance: 12 February 1996